MICROPROPAGATION OF *Curcuma xanthorrhiza* Roxb. AND *Zingiber aromaticum* Vahl. WITH ANTIMICROBIAL ACTIVITIES VIA AERATED CULTURE SYSTEM

by

MELATI YULIA KUSUMASTUTI

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FOR MY DEAREST FAMILY....

BANG JUNANDA

BAPAK & IBU

DEK TIKA & DEK DITO

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LIST OF ABBREVATION

BA	6-benzylaminopurine
IBA	Indole-3-butyric acid
g/L	Gram per litter
mg/L	Milligram per litter
MIC	Minimum Inhibitor Concentration
min	Minute
MS	Murashige and Skoog
NA	Nutrient Agar
PDA	Potato Dextrose Agar
SDA	Sabouraud Dextrose Agar
t_R	Retention time
v/v	Volume per volume
µg/mL	Microgram per mililitter
µL	Microlitter

MIKROPROPAGASI *Curcuma xanthorrhiza* Roxb. DAN *Zingiber aromaticum* Vahl. YANG MEMPUNYAI AKTIVITI ANTIMIKROB MELALUI SISTEM KULTUR PENGUDARAAN

ABSTRAK

Tunas rizom *Curcuma xanthorrhiza* and *Zingiber aromaticum* yang aseptik dapat dihasilkan melalui pensterilan permukaan dengan menggunakan 70% etanol selama 20 minit diikuti dengan 50% (v/v) Clorox® selama 20 minit. Prosedur ini menghasilkan 100% dan 83.3% tunas aseptik *C. xanthorrhiza* dan *Z. aromaticum* masing-masing. Kesemua tunas aseptik kedua-dua spesies bermandiri apabila dikulturkan ke dalam medium MS ditambah 0.5mg/L BA dan 0.5mg/L IBA. Bilangan pucuk yang terbentuk daripada setiap eksplan pucuk bagi kedua-dua spesies dengan menggunakan medium MS ditambahkan 1.0 mg/L BA (*C. xanthorrhiza* 3.7 pucuk/eksplan; *Z. aromaticum* 4.3 pucuk/eksplan) didapati tidak berbeza berbanding dengan MS ditambahkan 0.5mg/L BA dan 0.5mg/L IBA (*C. xanthorrhiza* 3.9 pucuk/eksplan; *Z. aromaticum* 3.0 pucuk/eksplan). Oleh itu medium MS ditambahkan 0.5mg/L BA dan 0.5mg/L IBA digunakan sebagai medium proliferasi pucuk untuk kedua-dua spesies. Walaubagaimanapun, pucuk dibahagi dua bagi *C. xanthorrhiza* dan *Z. aromaticum* masing-masing menghasilkan purata 6.3 dan 2.9 pucuk per eksplan (bersamaan dengan 12.6 dan 6.8 pucuk setiap pucuk lengkap) dengan menggunakan medium proliferasi pucuk. Bilangan pucuk yang terhasil daripada pucuk yang dibahagi dua adalah lebih banyak secara berarti berbanding pucuk lengkap. Sebanyak 524 dan 329 pucuk mampu diperolehi masing-masing daripada 200 pucuk dibahagi dua (bersamaan 100 pucuk lengkap) daripada *C. xanthorrhiza* dan *Z. aromaticum*. Pucuk eksplan *C. xanthorrhiza* dan *Z. aromaticum*

yang dikultur ke dalam medium proliferasi cecair menghasilkan lebih banyak pucuk berganda berbanding medium beragar. Keputusan yang diperolehi menunjukkan rizom yang dikutip dari lokasi yang berbeza tidak mempengaruhi kadar pembentukan pucuk berganda bagi *C. xanthorrhiza*. Semua eksplan pucuk *in vitro C. xanthorrhiza* dan *Z. aromaticum* (100%) menghasilkan akar di dalam medium asas MS pepejal empat minggu selepas pengkulturan. Anak benih *in vitro* ini ditanam dalam campuran tanah liat : pasir : tanah organik (1:1:1) di dalam dulang plastik selama dua minggu untuk aklimatisasi diikuti pemindahan ke beg politena dengan komposisi tanah yang sama selama lima minggu. Kadar peratusan kemandirian anak benih *in vitro* selepas proses aklimatisasi bagi *Curcuma xanthorrhiza* adalah 75.0% manakala 92.6% bagi *Zingiber aromaticum*. Pucuk mikro *C. xanthorrhiza* dan *Z. aromaticum* yang dihasilkan melalui sistem kultur pengudaraan menunjukkan peratusan mandiri yang rendah apabila dipindahkan secara langsung ke tanah. Namun sekiranya pucuk mikro dibiarkan berakar terlebih dahulu di dalam medium MS sebelum dipindahkan ke tanah, 80% hingga 93.3% anak benih dapat bermandiri. Ekstrak metanol rizom pokok induk dan anak benih *in vitro Zingiber aromaticum* mempunyai aktiviti antimikrob terhadap *Pseudomonosa aeruginosa* dengan MIC 1250 µg/ml (pokok induk) dan 625 µg/ml (anak benih *in vitro*). Ekstrak ini juga mempunyai aktiviti antimikrob terhadap *Escherichia coli* dengan MIC 625 µg/ml bagi kedua-dua pokok induk dan anak benih *in vitro*. Manakala, ekstrak metanol daripada rizom pokok induk, anak benih *in vitro* serta pucuk *C. xanthorrhhiza* yang diperoleh daripada sistem kultur pengudaraan tidak menunjukkan sebarang aktiviti antimikrob terhadap organisma mikrob yang diuji. Ekstrak metanol daripada anak benih *in vitro* bagi *C. xanthorrhiza* dan *Z. aromaticum* telah dianalisis dengan GC/MS. Kompund seperti (+)-a-ar-curcumene, dihydrocurcumene, xanthorrizol dan 2-methoxy-4-vinylphenol

telah ditemui bagi ekstrak metanol daripada anak benih *in vitro* bagi *C. xanthorrhiza*. Manakala 2-methoxy-4-vinylphenol, 1,3-propanediol, α-humulene, limone dioxide dan zerumbone telah ditemui bagi ekstrak metanol daripada anak benih *in vitro* bagi *Z. aromaticum*.

MICROPROPAGATION OF *Curcuma xanthorriza* Roxb. AND *Zingiber aromaticum* Vahl. WITH ANTIMICROBIAL ACTIVITIES VIA AERATED CULTURE SYSTEM

ABSTARCT

The aseptic rhizomatous buds of *Curcuma xanthorrhiza* and *Zingiber aromaticum* were established by surface sterilizing with 70% ethanol for 20 minutes followed by 50% (v/v) Clorox® for 20 minutes. This procedure produced 100% and 83.3% aseptic buds explants of *C. xamthorrhiza* and *Z. aromaticum* respectively. All the aseptic buds of both species survived when cultured on MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA. The number of shoots formed from each shoot explant for both species using MS medium supplemented with 1.0 mg/L BA (*C. xanthorrhiza* 3..7 shoots per explant; *Z. aromaticum* 4.3 shoots per explant) was found to be not significantly different when compared with cultures using MS supplemented 0.5 mg/L BA and 0.5 mg/L IBA (*C. xanthorrhiza* 3.9 shoots per explant; *Z. aromaticum* 3.0 shoots per explant). Hence MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA as the shoot proliferation medium was used for both species understudied. However, longitudinally cut shoots (half shoots) explant of *C. xanthorrhiza* and *Z. aromaticum* produced an average of 6.3 and 2.9 shoot per explant respectively (equivalent 12.6 and 6.8 shoot per whole shoot) using the shoot proliferation medium. The number of shoot produced from each half shoot explants was significantly higher than the whole shoot explants. A total of 524 and 329 shoots could be produced respectively from the *C. xanthorrhiza* and *Z. aromaticum* 200 half-shoot explants (equivalent to 100 whole shoots). Shoot explants of *C. xanthorrhiza* and *Z. aromaticum* cultured on liquid proliferation medium produced

more multiple shoots than the agar-gelled medium. Results obtained indicated that rhizomes collected from different locations did not affect the rate of multiple shoot formation for *C. xanthorrhiza*. All of the *in vitro* shoots of *C. xanthorrhiza* and *Z. aromaticum* (100%) produced roots in solid MS basal medium after four weeks of culture. These *in vitro* plantlets were planted in mixture of top soil : sand : organic soil (1:1:1) in plastic trays for two weeks for acclimatization followed by transferring to polythene bags containing the same soil composition for five weeks. The survival percentage was 75.0% for *Curcuma xanthorrhiza* and 92.6% for *Zingiber aromaticum* after the acclimatization process. The micro-shoots of *C. xanthorrhiza* and *Z. aromaticum* produced in the aerated flask system showed poor survival percentage when they were directly transferred to soil from the aerated flask system. However, if the micro-shoots rooted in MS basal medium before transferred to soil, 80 to 93.3% of the plantlets could survive. The methanol extract from the rhizomes of mother plants and the *in vitro* plantlets of *Zingiber aromaticum* exhibited antimicrobial activity against *Pseudomonosa aeruginosa* with MIC 1250 µg/ml (mother plant) and 625 µg/ml (*in vitro* plantlets). The extracts also have the same antimicrobial activity against *Escherichia coli* with MIC 625 µg/ml for both the mother plant and *in vitro* plantlets. While, the methanol extract of *C. xanthorrhhiza* from the mother plant rhizome, *in vitro* plantlets and shoots derived from the aerated flask system did not showed antimicrobial activity against the tested microbial organism. Methanol extract from the *in vitro* planlets of *C. xanthorrhiza* and *Z. aromaticum* were analyzed by GC/MS. Compounds such as (+)-a-ar-curcumene, dihydrocurcumene, xanthorrizol and 2-methoxy-4-vinylphenol were found in methanol exract from the *in vitro* plantlets of *C. xanthorrhiza*. While, 2-methoxy-4-

vinylphenol, 1,3-propanediol, α-humulene, limone dioxide and zerumbone were found in methanol exract from the *in vitro* plantlets of *Z. aromaticum*.

CHAPTER ONE

INTRODUCTION

1.1 Background

Microorganisms such as bacteria, fungi, archaea or protists survive almost everywhere on earth where there is moisture, including hot springs, the ocean floor and rock interior. There are friendly microorganisms that are critical to nutrient recycling in ecosystems. On the other hand, there are pathogenic microbes that can invade other organisms and cause many infectious diseases such as plague, tuberculosis, anthrax, malaria, sleeping sickness and toxoplasmosis; and fungi causing diseases such as ringworm, candidiasis or histoplasmosis.

Plants are one of the important sources of antimicrobial drugs, because in nature plants can produce antimicrobial substances that kill or inhibit the growth of microbes such as bacteria (antibacterial activity) (Taguri et al., 2004), fungi (antifungal activity) (Roberts and Selitrennikoff, 1988), viruses (antiviral activity) (Rudrappa and Bais, 2008), or parasites (anti-parasitic activity) (Anthony et al., 2005).

Plants produce over 100,000 secondary metabolites. Secondary metabolite is another component beside the components of intermediary (primary) metabolism which are generally nonessential for the basic metabolic processes of the plant. Most of them are derived from the isoprenoid, phenylpropanoid, alkaloid or fatty acid /polyketide pathways. Many products of secondary metabolites are used as defense agents against microbial attack or insect/animal predation (Bennett and Wallsgrove, 1994, Dixon, 2001). The major groups of antimicrobial compounds from plants are

phenolics, polyphenols, terpenoids, essential oils, alkaloids, lectins, polypeptides, and many other compounds (Cowan, 1999).

Many species of the Zingiberaceae family are being used in traditional medicine. They are found to be effective in the treatment of several diseases. The rhizomes of these Zingiberaceae plants have been used as antimicrobial (Konning et al., 2004; Tachakittirungrod and Chowwanapoonpohn, 2007), antiulcer (Borrelli and Izzo, 2000; Mahattanadul et al., 2009); anti-inflammatory (Ojewole, 2006), antioxidant (Selvam et al., 2005; Tachakittirungrod and Chowwanapoonpohn, 2007), cytotoxic and antitumor (Zhou et al., 1997; Lin et al., 2006; Zhao et al., 2008), vasorelaxant (Matsuda et al., 2001; Adaramoye et al., 2009), antispasmodic (Prakash et al., 2006), antihepatotoxic (Kim and Kim, 2004), and antidepressant agents (Yu et al., 2002), etc.

Recent study indicated that curcumin, the most active content and yellow pigment derived from *Curcuma longa*, has potential antibacterial activity against *Helicobacter pylori*. Curcumin was found to be highly effective in inhibiting *Helicobacter pylori* growth (De et al., 2009). Another prospective species reported to have antimicrobial activities are *Curcuma xanthorrhiza* and *Zingiber aromaticum*. Xanthorrhizol possess antibacterial activity against *Streptococcus* species that causes dental caries (Hwang et al., 2000). It was also reported that *Z. aromaticum* extract had potential activity in inhibiting the growth of *Helicobacter pylori* (Ekasari et al., 2001). *Z. aromaticum* had also been proven to inhibit human microsomal cytochrome P450 3A4 (CYP 3A4) and CYP2D6 which oxidized and metabolized the clinical used drug (Usia et al., 2005).

Most plants do not produce secondary metabolites such as curcumin, xanthorrhizol, and essential oil in large amount. *In vitro* Culture Techniques can be

used as alternatives for the production of secondary metabolite. Commercial scale *in vitro* propagation generally used gelled medium for proliferation of plants, but this requires large number of culture vessels, labour intensive with high energy consumption. This resulted in both low propagation efficiency and high production cost.

In order to solve these problems, large-scale propagation technique with simple culture protocol with fewer equipments and reduced production cost should be adopted. The use of bioreactor technique seems to be the most promising in reducing the labour cost and establishing a practical system for *in vitro* mass propagation of plantlets.

There are many types of bioreactors that can be used in plant *in vitro* culture techniques. They are classified as agitation methods (aeration-agitation bioreactors, rotating drum bioreactors, spin filter bioreactors), pneumatically agitated bioreactors (unstirred bubble bioreactor, bubble column bioreactor, air-lift bioreactor), and non-agitated bioreactors (gaseous phase bioreactor, oxygen permeable membrane aerator bioreactor, overlay aeration bioreactor) (Takayama and Akita, 1994). The most frequently used bioreactors that are suitable for plant organ cultures, especially for shoot cultures are unstirred bubble bioreactors, bubble column bioreactors and airlift bioreactors. But all these bioreactor are expensive and this contributes to the extra production cost. Hence, the present studies were proposed to study the possibility of using modified aerated culture vessel for mass propagation of Zingiberaceae species using *Curcuma xanthorrhiza* and *Zingiber aromaticum* as models and an efficient propagation protocol could be established.

1.2 Objective

The objectives of the study therefore were:

1. To study the propagation of *Curcuma xanthorrhiza* and *Zingiber aromaticum* using the conventional tissue culture technique.
2. To mass produce *Curcuma xanthorrhiza* and *Zingiber aromaticum* with a modified aerated culture vessel.
3. To compare the antimicrobial activity of the shoot culture of *Curcuma xanthorrhiza* and *Zingiber aromaticum* with their mother plants.

CHAPTER TWO

LITERATURE REVIEW

2.1 Zingiberaceae

2.1.1 Zingiberaceae family

Zingiberaceae family, also known as ginger family consists of 53 genera and more than 1200 species. This is the largest family in the order Zingiberales. Indo-Malaysian area is where the most genera and species of this family located (Sharma, et al., 2011). Approximately 330 to 350 species of Zingiberaceae representing 21 genera (including Costus) were found in East and West Malaysia (Ibrahim, 1991). This family can be divided into four subfamilies, two of which contain a single genus, and the other two larger subfamilies divided into two tribes. Subfamily Siphonochiloideae consist of *Siphonochilus*; subfamily Tamijioideae consist of *Tamijia*; subfamily Alpinioideae consists of tribe Riedelieae (*Burbidgea, Pleuranthodium, Riedelia, Siamanthus*) and tribe Alpinieae (17 genera); while Zingiberoideae consist of tribe Globbae (*Gagnepainia, Globba, Hemiorchis*) and tribe Zingiberae (26 genera) (Kress, 2002).

Most of them grow in damp and humid shady area, but some species can be fully exposed to the sun and grow in high elevation (Sirirugsa, 1999; Habsah et al., 2000). Zingiberaceae members are rhizomatous herbs. Some are very robust and contain volatile oil. They produce leaves that are composed of clasping sheath, ligule and blade. The sheath forms the pseudostem. The inflorescences are terminal on the leafy shoots or on separate, leafless shoot, spicate, racemose or made up of cincinni borne in the axils of bracts. The flowers are zygomorphic, bisexual or functionally male. Three more or less distinct teeth is fused with the calyx. Corolla is three lobeb.

The labellum is oblong to lobed and at the base of labellum, lateral staminodes are present which may be small or large and often petaloid. There is one fertile stamen at the opposite of the labellum. The anthers are with two thecae, while the gynoecium is syncarpous. The ovary is inferior with three locules. The style passing through between the anther thecae is not fused to anther. Punctuate stigma with axile placentation and the ovules can be few in number or many in each locule. Fruits are fleshy or dry, indehiscent (especially *Pleuranthodium* and *Riedelia*) or dehiscent. Seeds several to many per locule and cotyledon is single (Newman, 2007).

2.1.2 Genus *Curcuma* and *Zingiber*

Genus *Curcuma* is a member of the tribe Hedychieae belonging to the family Zingiberaceae. This genus contains about 70 species found in tropical and subtropical regions (Purseglove, 1975). This genus is distributed from India to Thailand, Indochina, Malaysia, Indonesia and Northern Australia. The genus *Curcuma* is easily identified by its florescence, a compound spike with distinguishable bracts, each holding two cincinnus. Each cincinnus has ten flowers, and is joined together to each other forming pockets at the base. The uppermost bracts or `coma' are usually the longest, and is differently colored and can be sterile. This genus flowers naturally from April to October. Flowering in genus *Curcuma* is marked by appearance of the inflorescence stalk which usually starts before the leaves comes out. The plants in genus *Curcuma* possess branched and fleshy rhizomes and the roots produce ellipsoid tubers. The large leaves are usually elliptically oblong. The height of the plant differs from 50 to 200 cm. Usually the plant species in genus *Curcuma* are difficult to distinguish among themselves as they

have similar growth habits, leaf shape and flowers during their reproductive and vegetative stages (Apavatjrut et al., 1999).

Genus *Zingiber* consists of 150 species. It was mainly distributed in South China, Malaysia, Northeast India, Myanmar and the Java-Sumatra provinces of Indonesia. Many species in this genus are grown in the garden as ornamental and as cut flowers in floral arrangements, while some of the species are used as valuable medicines. *Zingiber* plants are perennial herbs, medium-sized with stout aromatic rhizome. Rhizomes are normally close to the surface of the ground. The rhizome and pseudo stem of this genus are softer and thicker than other genus such as *Alpinia* and *Ammomun*. The inflorescences are produced on separate shoot directly from the rhizome. The flowers usually cross-pollinated and this is characteristic of this genus. The flowers are produced on broad or narrow, cone shaped or cylindrical spike which are overlapped closely and sometimes brightly coloured. The flowers arising from the bracts are short lived. The flowers produces thin calyx. The corolla tube and the bracts are of similar length. The petal is unequally broad which may be cream or white in colour. The beak like or horn shaped appendage of the anther make it unique. Every each species in this genus has different flowering time, but it is consistent for each species (Henderson, 1954; Ravindran et al., 2005).

2.1.3 *Curcuma xanthorrhiza* Roxb.

2.1.3.1 Morphology of *Curcuma xanthorrhiza* Roxb

Curcuma xanthorrhiza Roxb. is also known as “temulawak” in Indonesia and Malaysia. It is herbaceous plant with a pseudo green or dark brown stem. It can grow to a height of around two meters. The stem can reach up to 9 to 23 cm and 4 to 6 cm in width. Each stem consist of about two to nine leaves. The leaves are elongated

round, green or light brown to dark purplish, with a length of 31 to 84 cm with petiole length of (including the blade) about 43 to 80 cm. The branched rhizomatous roots are dark green in color. While, the rhizome is orange or yellow-brown to brown in color. The rhizome of *C. xanthorrhiza* has pungent and bitter taste with aromatic scent. Lateral staminodes, line-shaped scales and lean shaft are present. The flower produces bract with length much longer or equal to the flowers. The white-haired petals are about 8 to 13 mm in length. The flowers are tubular with a total length of 4.5 cm. There are also circular strand of white elongated flowers with a red tip with a length of 1.25 to 2 cm and 1 cm wide. The lips of the flowers are orange or red with a length of 14 to 18 cm and 14 to 20 mm wide. Light yellow stamens 12 to 16 mm in length and width of 10 to 15 mm. (Ridley, 1967).

2.1.3.2 Constituent of *Curcuma xanthorrhiza* Roxb.

The rhizome of *Curcuma xanthorrhiza* Roxb consists of 1–2 % yellow pigment, especially curcumin, mono- and bisde-metoxycurcumin and other related compounds that are not volatile in steam. It also contains sesquiterpenes, such as β-curcumen, arcurcumen and xanthorrhizol (Bisset and Wichtl, 1994). The rhizome also consists of high essential oils (7-11%). Camphor, *p*-tolyl, methyl carbinol, and myrceren are some of the compounds found in the oil (Attokaran, 2011). The ethyl acetate fraction of *Curcuma xanthorrhiza* extract contains curcumin, demetoxycurcumin and bisdemetoxycurcumin (Ruslay et al., 2007).

2.1.3.3 Uses of *Curcuma xanthorrhiza* Roxb.

Curcuma xanthorrhiza is one of the many well-known plants used as traditional medicine in Indonesia. As traditional medicinal herbs, *C. xanthorrhiza*

could be used as main ingredient, supplement ingredient, coloring agent and flavouring agent. Boiled and brewed rhizomes that have been made into powder could be used to treat liver disease or jaundice (Dalimartha, 2006). *C. xanthorrhiza* has been used as choleretic and cholekinetic in treating chronic forms of cholingitis and cholecystitis, as well as gallstones. The essential oil of *C. xanthorrhiza* was responsible for the choleretic action, while the curcumin might be responsible for the cholekinetic effect. It was also used as stomachic and carminative agents (Bisset and Wichtl, 1994). Curcumin produced in *C. xanthorrhiza* is used for increasing the contraction of the gall bladder. This activity suggested that this chemical might be useful in preventing gall bladder stone formation (Rasyid et al., 2002). Beside that curcumin also has been proven to have antioxidant activity, antiinflamatory activity and inhibits the carcinogenic-DNA and tumorigenesis (Lin, 2004). Xanthorrizol, isolated from the methanol extract of *C. xanthorrhiza*, was reported to have antimicrobial activity against bacteria *Streptococcus* species that caused dental caries (Hwang et al., 2000). This compound also reported to have anti-metastatic activity in experimental mouse lung metastasis model (Choi et al., 2004).

2.1.4 *Zingiber aromaticum* Vahl.

2.1.4.1 Morphology of *Zingiber aromaticum* Vahl.

Zingiber aromaticum Vahl. is also known as “lempuyang wangi” in Indonesia, “lempoyang wangi” in Malaysia or “fragrant ginger” in English. It is considered to be native of Asia. It has a pseudo stem with a height around 1.0 to 1.5 m. Leaves are long and lancet-shaped with 14 to 40 cm length and 3 to 8.5 cm width. The base of the leaf may be round or sharp or pointed. The upper leaf surface is hairy. Hairy petiole with a length of 4 to 5 mm. Petals are round shaped shorter than

the bracts of the flower. Bracts with a length of 1.5 cm to 4 cm and wide 1.25 to 4 cm. Crown flowers are bright yellow, dark yellow or yellowish white produced in a red cone arising from the base of the plant. The rhizome is strongly aromatic (Anonymous, 1983; Sabu and Skinner, 2005).

2.1.4.2 Constituent of *Zingiber aromaticum* Vahl.

Volatile oil (0.5-1%), zerumbone, humulena, limonene are compounds that are found in the rhizome of *Z. aromaticum*. The methanol extract of *Zingiber aromaticum* Vahl. rhizome consist of a new sesquiterpene [2,9-humuladien-6-ol-8-one], three known humulane-type sesquiterpenes (zerumbone, zerumbone epoxide, tricyclohumuladio), three gingerols ((*S*)-6-gingerol, (*S*)-8-gingerol, (*S*)-10-gingerol), two shogaols (*trans*-6-shogaol, *trans*-10-shogaol), two flavonoids (kaempferol-3-*O*-methyl ether, kaempferol-3,4'-di-*O*-methyl ether), two acetylated-flavonol-glycosides`(kaempferol-3-*O*-(3-*O*-acetyl-a-L-rhamnopyranoside), kaempferol-3-*O*-(4-*O*-acetyl-a-L-rhamnopyranoside)), kaempferol-3-*O*-a-L-amnopyranoside, b–sitosterol and b-sitosterol glucoside. Among the known compounds, trycyclohumuladiol, (*S*)-8-gingerol, (*S*)-10-gingerol, *trans*-10-shogaol, and b-sitosterol glucoside were detected for the first time in this plant (Usia et al., 2004).

2.1.4.3 Uses of *Zingiber aromaticum* Vahl.

Zingiber aromaticum is widely grown as the kitchen garden and ornamental plant. The shoots and the flowers are eaten and used to flavor food. The rhizome are traditionally used as analgesic, for the treatment of asthma, oedema after giving birth, intestinal worm, cholera, anemia, influenza, malaria, colds, vomiting, appetite enhancer, abdominal pain, rheumatic, stomach ulcer, shortness of breath,

constipation, tuberculosis, hemorrhoids and liniment for warming the bodies (Hariana, 2008). Research conducted by Ekasari (2001) indicated that among several species of Zingiberaceae family, the extract of *Z. aromaticum* is the one that has the activity in inhibiting the growth of *Helicobacter pylori*. *Z. aromaticum* had also been proven to inhibit human microsomal cytochrome P450 3A4 (CYP 3A4) and CYP2D6 which oxidized and metabolized the clinical used drug (Usia et al., 2005). Zerumbone which is the active compound from *Z. aromaticum* had also been proven to inhibit the growth of three human cancer cell lines; HT-29 colon, CaCo-2 and MCF-7 breast cancer cell (Kirana et al., 2003). Dai (1997) reported that zerumbone isolated from organic extract of rhizomes of *Z. aromaticum* and *Z. zerumbret* showed HIV-inhibitory and cytotoxic activities. The flavonol glycosides (kaempferol-3-*O*-(2,3,4-tri-*O*-acetyl-R-Lrhamnopyranoside) and kaemferol-3-O-methyl ether) isolated from the water extract of Z. *aromaticum* have inhibitory activities against CYP3A4 and CYP2D6 (Usia et al., 2004).

2.2 *In vitro* propagation of plantlets

2.2.1 Micropropagation

Micropropagation is an *in vitro* culture technique that is commonly used as the true-to-type propagation of selected genotypes. This technique can be achieved via various ways, such as shoot proliferation (shoot culture), node culture, de novo formation of adventitious shoots through shoots organogenesis or somatic embryogenesis, depending on the plant species and culture conditions (Sathyanarayana and Varghese, 2007). This technique has been used as one of the alternatives for plant breeding and propagation. Microprogation has many advantages which inculde rapid clonal multiplication of plantlets, especially the

endangered species and economic crops recovery of disease-free clones and conservation of germsplasm, genetic improvement of crops and production of pharmaceutical or other natural product (secondary metabolites) in medicinal plant (Liao et al., 2006; Yoshimatsu, 2008; Singh and Kumar, 2009).

There are five stages (stage 0 to IV) in micropropagation of plant species. It starts with Stage Zero, the selection of mother plant's part and preparation of stock plant. In Stage One, the establishment of aseptic culture, the chosen plant parts are surface-sterilized and cultured aseptically into culture medium. Stage Two is the multiplication of propagules. Stages Three is the rooting of the micro-shoots and prepare them for growth in the natural environment. Stage Four is transferring the plantlets to the natural environment and known as the acclimatization process (George et al., 2008).

Stage Zero - Donor plant or mother plant selection and preparation

Selection of the stock plants is highly recommended before micropropagation commences. They should be free from any symptoms of disease. Maintenance of the selected stock plant (or part of it) is to ensure the success of the *in vitro* cultures. The right environment and appropriate chemical pre-treatment of stock plants can enhance the growth, morphogenesis and rates of propagation. Procedures to detect and reduce or eliminate systemic bacterial and viral diseases may also be required. Disease indexing and disease elimination should be an important part of the micropropagation process.

Stage One – Establishment of aseptic culture

The purpose of this stage is to obtain the aseptic culture. The culture should be free from any pathogenic disease. The success at this stage is indicated by the non-contaminant explants after being transferred to the culture medium and show

response to growth. The choice of suitable surface-sterilization method is important for this stage. There are several sterilizing agents that can be used for surface-sterilization of plant explants, such as calcium hypocloride, sodium hypochloride (NaOCl), mercuric chloride, hydrogen peroxide, silver nitrate, bromine water, ethyl alcohol. Sometimes antibiotic (e.g. penicillin-G, bacitracin, terramycin, oxytetracycline, rifampicin and gentamycin) and polysan (germicide detergent) are required to establish aseptic explants. Calcium and sodium hypochloride are commonly used as disinfectant agents because they are harmless and effective for eliminating bacterial contamination (Narayanaswamy, 1994). Combination of $HgCl_2$ solution and Clorox® were used to surface sterilize the bud explants of some plant species of the Zingiberaceae family (Chan and Thong, 2004). Calcium hypocloride solution and 70% alcohol were used to establish the aseptic cladode explants of *Opuntia amyclaea* (Escobar et al., 1986).

Stage Two – Multiplication of propagules

The objective of Stage II is the multiplication of new propagules. Multiplication can be performed from newly formed axillary or adventitious shoots, somatic embryos, or propagative organs. Induction of meristematic tissue at the adventitious organs may is included in this stage. Some of the propagules that produced at Stage II (especially shoots) could also be used as the basis for further multiplication cycles. They are usually subcultured to increase the numbers.

Stage III – Rooting process

The micro-shoots that derived in Stage II are normally small and not able to survive when transferred to outside environment. Hence rooting in stage III is needed for formation of plantlets. Some plantlets need special treatment at this stage, so that their growths are not inhibited or become dormant when taken out of the *in vitro*

environment. The very important step of any *in vitro* propagation process is the rooting process. During Stage III, some plant species could form adventitious roots easily, but some need to adopt a special rooting procedure using special medium, or methods, to induce root formation. Sometimes shoot elongation need to be carried out first before the rooting procedure. Sometimes the unrooted micro-shoots need to undergo the rooting process outside the vessel.

Stage IV – Acclimatization process

Acclimatization is a process that enables the *in vitro* plantlets to grow successfully when transferred from the *in vitro* condition to the external environment. This process is very important and if not done carefully could result in significant loss of propagated plantlets. At this stage, the plantlets were taken out from the culture vessels after rooting. Agar is carefully cleaned or washed if they were grown on agar medium. The leaves can be applied with anti-transpirant film which is not usually used. The *in vitro* plantlets can be transferred to a suitable growing substrate, such as a peat, sand compost and stored in high humidity and less light intensity conditions for a few days. Humidity can be maintained by fog of water vapour. Water misting intermittently is an alternate way of maintaining the high humidity. For some plant species, Stage III can be avoided and the *in vitro* shoots can be directly rooted in high humidity and gradually hardened to the outside environment.

2.2.2 Composition of culture medium

The selection of suitable medium is related with the success of the micropropagation technique. Various basal media have been used for plant *in vitro* cultures, but Murashige and Skoog (MS) (1962) basal medium is the most commonly

used with or without modification. Nutrient medium basically consists of inorganic salt (macro and micro nutrient), vitamins, plant growth regulators, and a carbohydrate as carbon source or gelling agent. Other compounds such as organic nitrogen compound, hexitol, amino acids, antibiotic and other plant extracts can be included in medium as optional additives. The formulation and composition of a nutrient medium depend on plant species and the objective of culture technique (Singh and Kumar, 2009).

The macronutrients consist of six elements: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulphur (S) present as salt in the culture medium. Any plant cultures need all the macronutrient elements, but the optimal concentration of the macronutrients is different depending on the plant species (Razdan, 2003). Micronutrients are inorganic elements with addition of small amount in the culture medium but they are essential for the growth of plant cells and tissues. Iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), chloride (Cl), molybdenum (Mo), cobalt (Co) and iodine (I) are the common micronutrients used in a culture medium. Other elements such as aluminium (Al) and nickel (Ni) are rarely used. Iron can be considered as the most important micronutrient because the quantity of iron is the highest among other micronutrients. Iron chelated with EDTA was usually used to avoid precipitation and facilitate absorption. (Yadav and Tyagi, 2006; Sathyanarayana and Varghese, 2007).

Sugar is used as the energy supply in a culture medium. Sucrose is the most commonly used. Other carbohydrates such as glucose, fructose, sorbitol, and maltose have also been used. Sucrose increases the potential osmotic when it is hydrolyzed into glucose and fructose after autoclaving. Normally 2% to 6% sucrose

is added into medium and used as carbon and energy source for tissue or plant growth (Beyl, 2010[b]).

Vitamins are important for plant growth. Thiamine (B1), pyridoxin (B6) and nicotinic acid (B3) are usually added into the culture medium. But other vitamins such as biotin, folic acid, ascorbic acid (vitamin C) and tocopherol (vitamin E) are sometimes used in medium formulation. Besides vitamins for enhancement and support the growth of plant tissue culture, myo-inositol is also added into most media formulation (Cardoza, 2008). Amino acids are also important for the growth of plant tissue especially in morphogenesis. The commonly used amino acids are all L-forms of amino acids, such as L-tyrosine, L-arginine, L-serine, L-cysteine, L-glutamine and L-asparagine (Singh and Kumar, 2009).

One of the factors that influence the uptake of various elements in the medium is pH of the medium. It also affects the regulation of biochemical reaction that occurs in plant tissue culture. Commonly the pH of the medium was adjusted to 5.7-5.8 for most of the culture media. Acidic media delay the growth of contaminants and do not have negative effect on the plant tissue culture (Cardoza, 2008). For solid or semi solid culture media, agar is the most commonly used. However, other gelling agents like gelatine, agarose, alginate or gelrite are used occasionally (Rout et al., 2000).

2.2.3 Plant Growth Regulators (PGRs)

Plant Growth Regulators (PGRs) added into the solid or liquid medium is important for the development of shoots, roots and embryos. Usually they help in cell division and expansion. Although plant cells can produce its own PGRs, supplement of PGRs into the medium is essential for the development of cells and organs.

Auxins and cytokinins are the most important PGRs used in tissue culture technology.

The important functions of auxins are involved in the development processes such as cell elongation and swelling of tissue, apical dominance, adventitious root formation and somatic embryogenesis. Depending on the concentration of auxins in the culture media, roots and callus can be formed. The most common auxins used are 1-naphtaleneacetic acid (NAA), 2.4-dichlorophenoxyacetic acid (2,4 D), 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) and indoleacetic acid (IAA) and indolebutyric acid (IBA). IBA is normally produced in higher plants naturally. Picloram and 2,4-D are widely used primarily to induce and regulate somatic embryogenesis. IAA, IBA and NAA are used for root formation

Cytokinins initiate cell division and shoot development. The cytokinins most commonly used are zeatin, dihydrozeatin, kinetin, benzyladenine (BA), thidiazuron and 6-(gamma, gamma-dimethylallyamino)purine (2iP). In higher concentration cytokinins can induce adventitious shoot formation but inhibit root formation. They are known to induce axillary shoot formation by suppressing the apical dominance control by auxins. The highest cytokinin activity is often showed by BA.

Gibberellins (GA) are least used PGR in plant tissue culture. GA is heat sensitive and after autoclaving 90% of the biological activity would be lost. Typically, it is filtered sterilized (Beyl, 2010[a]).

2.3 Secondary Metabolites

Plants are important source of chemical compounds of different classes for different uses. Chemical compounds that are produced by plants can be divided into two main classes, primary and secondary metabolites. Primary metabolites are

compounds that are essential for basic metabolic activities such as, proteins, amino acids, sugar, lipids and nucleic acids. Secondary metabolites are chemical compounds which are produced as by products of primary metabolites, but they are not essential for supporting the growth of plants. Alkaloids, flavonoids, tannins, terpins, essential oil and latex are some secondary metabolites that present in the plants (Nair, 2005).

The exact functions of secondary metabolites compounds are not understood clearly. Some compounds possess activities effective against fungus, bacteria or other pathogens. Some act as repellent to protect themselves from animal attack. Secondary metabolites have been used as dyes (indigo, shikonin), flavors (vanillin, capsaicin, mustards oil), fragrances (rose oil, lavender oil and other essential oil), stimulants (caffeine, nicotine, ephedrine), hallucinogens (morphine, cocaine, scopolamine, tetrahydrocannabinol), insecticides (nicotine, piperine, pyrethrin, rotenone), vertebrate and human poisons (coniine, strychnine, aconitine, colchicines, cardiac glycosides), and as therapeutic agents (atropine, quinine, cardenolides, codeine) (Wink, 2010).

Plants produce over 100,000 secondary metabolites. Most of them are identified as three major groups, alkaloid (12,000), phenolic (10,000) and terpenoid (25,000) (Bowsher et al., 2008).

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2.3.1 Alkaloids

These compounds are the most extensively studied compounds among the various groups of secondary metabolites. Approximately, more than 12,000 alkaloids have been isolated since the discovery of morphine (Croteau et al., 2000). Alkaloids are defined as organic molecules consisting of several ring structures that contain one

or more nitrogen atoms that are usually but not always located on a carbon ring. The structures of the carbon ring are usually used to classify these compounds (Hesse, 2002). Based on Saxena (2007), alkaloids are classified into several main groups: phenylethylamine, pyrrolidine, pyridine, piperidine, pyrrolidine-piridine, quinoline, isoquinoline, phenanthrene and indole alkaloids group. The most important biological function of alkaloids is protection of the plants cell against pathogen and herbivore, protection of generative reproduction, stimulation of growth and adaptation to local environment (Aniszewski and Ebrary, 2007). Alkaloids have been used as drugs, potions, medicines, tea, poultices and poisons for 4000 years. The first crude drug (alkaloid) investigated was opium (Roberts and Wink, 1998). Some plant alkaloid, such as stephanine and crebanine have been proven to possess antimicrobial activity (Deng et al., 2011), mytraginine reported to have analgesic activity (Watanabe et al., 1997), isoquinoline has been proven to have antiprotozoa activity (Tempone et. al., 2005).

2.3.2 Phenolic

Phenolic compounds are produced by the plants in many different types. To date there are about 10,000 different kinds of plant phenolic compounds that have been identified. Phenolics are a diverse group of compound with the common structure of aromatic hydrocarbon ring (phenyl or benzyl ring) which is usually attached to at least one hydroxyl structure. These compounds are divided into several classes: simple phenolics, phenolic acids and aldehydes, acetophenones and phenylacetic acids, cinnamic acids, coumarins, flavonoids, biflavonyls, benzophenones, xanthones, stilbenes, benzoquinones, anthraquinones, naphtaquinones, bethacyanins, lignans, lignin, tannin and phlobaphenes. The

flavonoids are classified again into several gruops: chalcones, aurones, flavanones, flavonols, flavones, leucoanthocyanidins, anthocyanidins, deoxyanthocyanidins, and anthocyanins (Vermerris and Nicholson, 2006). The functions of phenolics are as scent and pigment and protections against pathogen and predator. Interest in biological activity of phenolic compounds (especially flavonoids) from plants has recently been increased, mainly due to the potential health benefits associated with some dietary polyphenol (Pietta et al., 2003).

2.3.3 Terpenoids

Terpenoids represent the largest class of natural products. About 25,000 different forms of the compounds are already identified in higher plants. Terpenoids are various groups of essential oils that are formed from the fusion of five-carbon isoprene units. Terpenoids are classified according to the number of five-carbon units that they are formed. The 10-carbon terpenoids were called monoterpenes (i.e. single terpenes) despite the fact that they consist of two five-carbon units. The simplest terpenoids were called half terpenes or hemeiterpenes, which is consist of just one isoprene unit. There are sesquiterpenes (15-carbon terpenoids), diterpenes (20-carbon), triterpenes (30-carbon) and tertraterpenes (40-carbon). While the larger polymer is classified as polyterpenes, consist of eight or more five-carbon units (Bowsher et al., 2008). The terpenoids have diverse functional roles in plant such as hormonal function (gibberillin, abscisic acid, brassinosteroids), photosynthesis pigment (phytol, caretenoids), electron transport (ubiquinones, plastoquinones, menaquinones), mediator polysaccharide assembly (polyprenyl phosphates), and structural component of membranes (phytosterol) (McGarvey and Croteau, 1995). In addition, they also have specific functions in plant defense mechanism (phytoalexins,

insect feeding and oviposition deterrent and phytotoxin) and reproduction (attractant of pollinators and seed dispersing animals) (Pichersky and Gershenzon, 2002). These compounds are also commercially important as they are used widely in large number of industrial products such as flavouring agent, pharmaceutical product, perfumes, insecticides and antimicrobial agents (Aharoni et al., 2006).

Many secondary metabolites used as protection against microbial attack or insect/animal predation (Dixon, 2001). The major groups of antimicrobial compounds from plants are phenolics, polyphenols, terpenoids, essential oils, alkaloids, lectins, polypeptides, mixtures of other compounds (Cowan, 1999).

2.4 Bioreactor

Micropropagation technique using conventional gelled medium require large number of small containers and gelified media. It becomes a complicated and costly production technology. Periodic transfers of plant material to fresh media are normally carried out every 4 to 6 weeks, due to reduction of nutrients in the medium or continuous proliferation result in space limitation in the culture container (Maene and Debergh et al., 1985). Ornamentals, foliage plants and selected fruit crops produced via high production costs generally limit the commercial use of micropropagation (Simonton et al., 1991). Most expensive part of micropropagation process are cutting and inoculating the culture, while the labour costs are generally high as well, constituted from 40% to 60% of production costs (Chu, 1995).

Bioreactors are vessels designed for large-scale production of cells, tissues or organ cultures using liquid medium as culture medium. This system has shown several important advantages for increasing number of plantlets, among them are the increase in the multiplication rates and reduction in the use of space, energy and

labor cost (Levin and Tanny, 2002; Takayama and Akita, 2005). Bioreactors have other advantages, such as easier control of the culture conditions; supply of nutrients and growth regulators can be optimised; culture atmosphere can be renewed; culture medium can be changed during the culture period depending on the developmental stage; filtration of the medium for exudates; control of contamination; and bud or somatic embryo production for automated handling of the propagules (Ziv, 2005). In addition, bioreactor produced plant materials are normally physiological uniform; free of pathogens; produced high quality flowers; secondary metabolites and essential oil and reduced labour cost (Sivakumar et al., 2005).

Liquid media used in the process of micropropagation is very appropriate to be used to reduce the cost of production. The use of liquid culture systems could provide a more uniform culture conditions, media could be easily renewed without having to change the container and sterilization process much easier because it can be done by microfiltration. Compared with the semi-solid media, this system can be implemented by using a larger container and transfer time can be reduced (Ettienne and Berthouly, 2002).

There are two types of bioreactors. Cultures could be continuously submerged or temporarily immersed in the proliferation depending on the bioreactor type (Ducos et al., 2009). There are also some bioreactor that can be used for mass propagation of plant cells, tissue and organs, such as stirred tank bioreactor, bubble column, airlift bioreactor, and the rotating drum bioreactor (Honda et al., 2001). In general, bioreactors are grouped according to agitation method and vessel construction (Paek et al., 2005; Takayama and Akita, 1994).

The simple aeration/unstirred bubble bioreactors, bubble columns bioreactor and airlift bioreactor were the most frequently used for mass propagation of plant

organs, especially shoots cultures (Takayama and Akita, 2006). Mixing process in airlift bioreactor was done without mechanical agitation. Airlift bioreactors were used for mass propagation of tissues that were sensitive with mechanical shear. Usual mechanism of airlift was the air injected into the bottom of central draught tube through a sprager ring. The air flows through the draught tube to the head space of the bioreactor, where the excess air and any by-product, CO_2 are discharged. (Najafpour, 2006).

There were some benefit using airlift bioreactor, such as low shear stress, design and construction are simple, low consumption of energy and suitable for long term culture with minimum contamination (Paek et al., 2001).

2.5 Antimicrobial activities of plant extract

One of the diseases that caused morbidity and mortality in humans are infectious diseases. This happens, especially to citizens of under developed or developing countries. A lot of antimicrobial drugs have been developed and produced by pharmaceutical industries to prevent and cure the diseases. Most of them contain synthetic compounds as ingredient. However, the increases in resistance of microorganisms to these drugs have been observed. Micro-organisms like bacteria have the capability to acquire resistance to therapeutic drugs (Nascimento et al. 2000).

Fabricant and Farnsworth (2001) stated that only 6% of the plant population have been screened for biology activity and 15% have been evaluated for their phytochemicals content. The antimicrobial compounds derived from plants have different mechanisms to inhibit the growth of bacteria. By using active compounds of plants that have antimicrobial activity as antibiotics instead of the synthetic

compounds could give clinical value to treatment of the disease that were caused by resistant microbial strains (Eloff, 1998).

To date, many researches have been conducted on medicinal plants as alternative medicine for treating diseases caused by microorganisms. The diethyl extract of *Lonicera caprifolium* L., *Nepeta cataria* L., *Phytolacca dodecandra* L. and *Plantago lanceolata* L. have been proven to have activity against Gram positive bacterial and fungal organisms (Nostro et al., 2000). The combination extracts of the fruits of *Cornus officinalis* Sieb., *Cinnamomum cassia* and Chinese chive (*Allium tuberosum*) in the ratio of 8:1:1 was found to have antimicrobial activity for food borne microorganism (bacteria, yeast and moulds) (Hsieh et al., 2001). Aqueous crude extracts of *Curcuma longa* and *Zingiber officinale* showed broad spectrum antimicrobial activity and they could inhibit the growth of *Chromobacterium*, *Escherichia coli*, *Enterobacter faceales*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonela partyphii*, *S. thypii*, *Bacillus subtilis* and *Staphylococcus aureus* (Srinivasan et al., 2001).

The bioactive compounds of plants that have antimicrobial activity can be extract by various solvent. The water and methanol extracts of some Indian medicinal plants have shown antimicrobial activity against gram positive and negative bacteria (Parekh and Chanda, 2007). Ethanol extract of *Launaea procumbens* and *Cyperus rotundus* were active against *Alcaligenes faecalis*, *Bacillus cereus*, *B. subtilis*, *Enterobacter aerogenes*, *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa*, *P. pseudoalcaligenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *S. epidermidis*, *S. subfava* and *Candida tropicalis* (Parekh and Chanda, 2009). Some extract of South African plants, such as *Allium sativum*, *Tulbaghia violacea*, *Polygalamyrtifolia* and *Glycyrrhiza glabra*)